

Role of Prostaglandin-H Synthase in Mediating Genotoxic and Carcinogenic Effects of Estrogens

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Diethylstilbestrol (DES) has been found to be oxidized in Syrian hamster embryo (SHE) cells by prostaglandin-H synthase (PGH synthase). It is hypothesized that PGH synthase mediates adverse effects of DES and other carcinogenic estrogens such as induction of neoplastic transformation and genotoxicity. Interest in PGH synthase-catalyzed reactions focuses on two aspects: oxidation and metabolic activation of stilbene and steroid estrogens by PGH synthase, and modulation of prostaglandin biosynthesis via effects of these compounds on PGH synthase. Studies of the former aspect of PGH synthase-catalyzed *in vitro* metabolism have revealed that cooxidation of DES, DES analogues, and steroid estrogens gives rise to reactive intermediates; DES and DES analogues known to transform SHE cells are metabolized by PGH synthase *in vitro*; PGH synthase catalyzes both the formation and oxidation of catechol metabolites from steroid estrogens, and reactive intermediates from DES and from steroid estrogens are stable enough to bind both to the catalytic enzyme PGH synthase and to other proteins. The data support the contention that PGH synthase-catalyzed metabolic activation plays a role in the induction of neoplastic transformation by stilbene and steroid estrogens but is not conclusive evidence for a cause-effect relationship. More recently, two closely related DES indanyl analogues have been found to differ in their interaction with PGH synthase: indenestrol A is cooxidized and activated like DES, whereas indenestrol B inhibits the enzyme. They provide useful tools to test the above hypothesis from a new perspective. The interaction between estrogens and PGH synthase is also viewed with respect to its potential role in tumor promotion and progression, processes in which prostaglandins have been implicated as important mediators. Cooxidation of estrogens *in vitro* is accompanied by a stoichiometric increase in prostaglandin production. Useful approaches to study this and other effects of estrogens on PGH synthase that could result in a modulation of prostaglandin biosynthesis are discussed.

Introduction

Estrogens with documented carcinogenic activity *in vivo* can neoplastically transform cells in culture and induce genotoxicity in some *in vitro* systems (1). Diethylstilbestrol (DES), several DES analogues, and steroid estrogens have been found to induce neoplastic transformation of Syrian hamster embryo fibroblasts (SHE cells) (2,3) and of Balb/c 3T3 cells (4). For a number of these compounds, the neoplastic activity *in vitro* or *in vivo*, e.g., in the hamster kidney tumor model, did not correlate with hormonal activity, suggesting that their estrogenicity is not the whole explanation of their carcinogenic properties. Metabolic activation has been proposed to play an important role in the mechanism of action of DES and of steroid estrogens (5-7) but whether it is required is still an unresolved question.

In SHE cells, DES is metabolized by prostaglandin-H synthase (PGH synthase), an enzyme also present in other target cells (8). Furthermore, induction of sister

chromatid exchange by DES in certain cells is inhibited by indomethacin (9,10), a well-known PGH synthase inhibitor. These observations stimulated our interest in the PGH synthase-catalyzed oxidation of carcinogenic estrogens and its possible role in the process of neoplastic transformation induced by these compounds.

PGH synthase, a key enzyme in the biosynthesis of prostaglandins (PG) has both cyclooxygenase and peroxidase activity and catalyzes the oxygenation of arachidonic acid to the hydroperoxy endoperoxide PGG₂ and its reduction to the hydroxy endoperoxide PGH₂. The peroxidase reaction is accompanied by oxidation of (reducing) co-substrates (cooxidation). The PGH synthase-mediated *in vitro* oxidation of many xenobiotics yields electron-deficient, reactive intermediates, and techniques have been developed to estimate its *in vivo* contribution to the metabolic activation of chemical carcinogens (11). We have studied the oxidation of DES and its structural analogues as well as the PGH synthase-catalyzed metabolism of steroid estrogens and their catechol metabolites.

It is also of interest to establish whether the inter-

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action between PGH synthase and estrogens could result in a modulation of prostaglandin biosynthesis. Prostaglandins (in particular PGE₂) have been implicated as important mediators in tumor promotion and progression (12). That PGH synthase and its products may well play a role in hormonal carcinogenesis is suggested by observations that many tumors in estrogen target tissues progress under the influence of unsaturated precursor (ω -6) fatty acids, that murine mammary tumor growth can be inhibited by indomethacin (13-15), and that human breast epithelial cells proliferate in response to PGE₂ (16). Cyclic variations in PG biosynthesis occur in estrogen target tissues (17,18) so that a better understanding of the regulation and inducibility of PGH synthase by sex hormones seems to promise new insights in this field.

Methods

PGH Synthase-Catalyzed Metabolism of DES and Related Compounds

DES, DES analogues (Fig. 1), and steroid estrogens were incubated with microsomal or purified PGH synthase preparations in the presence of arachidonic acid (ARA) or peroxides (15-HPETE or H₂O₂); the conversion of estrogen parent compounds and the formation of products was assessed by HPLC analysis of incubation extracts as previously described in detail (19-21). Aryl hydroxylation of estrogens was determined in incubations with regiospecifically tritiated estradiol (E₂)

or DES by the tritium water release assay and by product identification (21,22).

Protein Binding of Reactive Estrogen Intermediates

Nonextractable binding to microsomal protein was found upon oxidation of radiolabeled estrogens by PGH synthase (23). The nature of the protein binding has been further studied in a refined *in vitro* system: estrogens, radiolabeled in a metabolically stable position, were activated by purified PGH synthase in the presence of various amounts of albumin (BSA) and/or tubulin. The amounts of radioactivity covalently bound to individual proteins were determined after SDS-gel electrophoresis by autoradiography and by combustion of excised gel bands followed by liquid scintillation counting (LSC) (21).

Effects of Estrogens on Prostaglandin Synthesis *In Vitro*

PGE₂ synthesis from radiolabeled arachidonic acid was measured in PGH synthase incubations in the presence of epinephrine (as reducing co-substrate) and various concentrations of DES indanyl analogues or indomethacin (Fig. 2). Inhibitory or stimulatory effects on arachidonic acid metabolism were also studied using the cyclooxygenase assay (20,24).

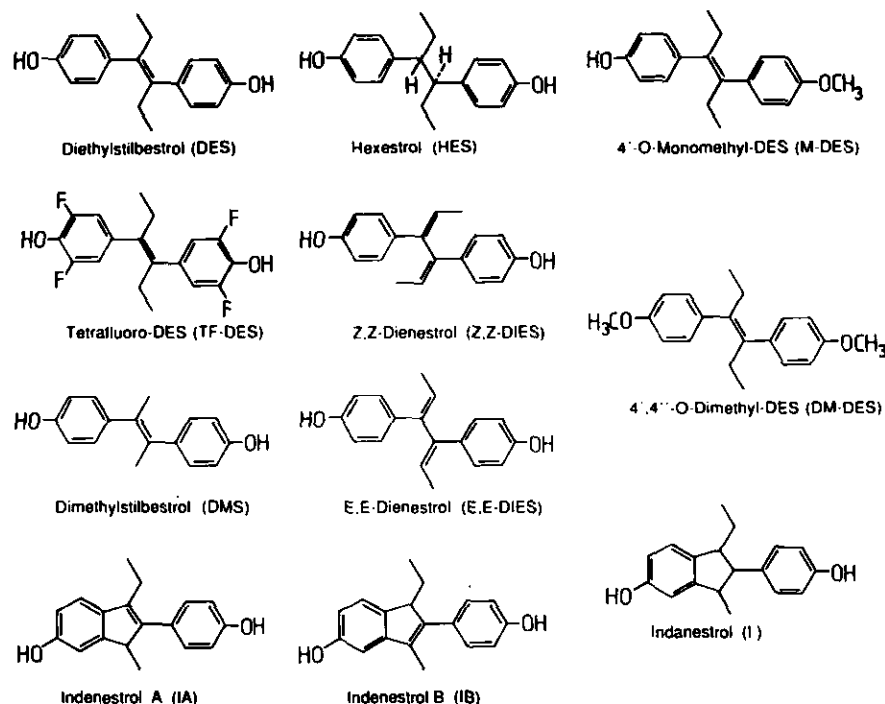


FIGURE 1. Structures of DES and related compounds.

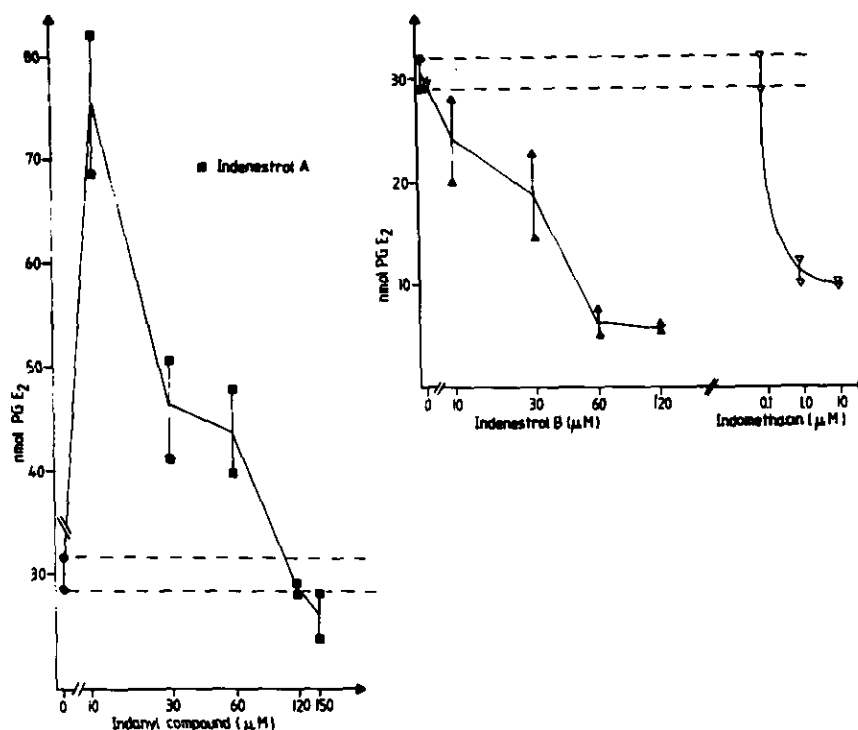


FIGURE 2. PGE₂-synthesis *in vitro* in the presence of test compounds indenestrol A, indenestrol B, or indomethacin. Microsomal PGH synthase (0.12 mg RSVM protein in 2.0 mL phosphate buffer, 0.05 M, pH 7.5, 1.0 mM epinephrine, and 0.5 mM GSH) was incubated with ¹⁴C-arachidonic acid (100 μM) and the indicated concentrations of test compounds for 5 min at 37°C. PGE₂ synthesis was determined in incubation extracts by radio-TLC as previously described (28). Dotted lines indicate the range of PGE₂ synthesis in controls with epinephrine (1.0 mM) and GSH (0.5 mM) but without addition of test compounds.

Results and Discussion

Metabolism of DES and DES Analogues by PGH Synthase

PGH synthase oxidizes DES *in vitro* by means of its peroxidase activity, predominantly to Z,Z-dienestrol (Z,Z-DIES), a metabolite also formed from catalysis with other peroxidases (5,19,25). The closely related derivatives, TF-DES and DMS (Fig. 1) are also oxidized via *para*-quinoid intermediates to the corresponding dien products (26).

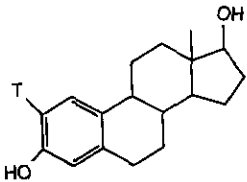
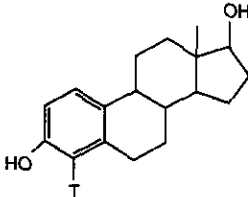
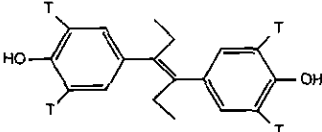
Other DES analogues without the conjugated double bond but with free phenolic groups (e.g., HES; E,E-DIES) are metabolized by PGH synthase via one-electron oxidation, primarily to phenoxy radicals (20). Cooxidation of DES and related compounds is accompanied by the formation of protein-bound products indicative of reactive (quinone or radical) intermediates. This makes PGH synthase a candidate enzyme for the metabolic activation of stilbene estrogens. DES oxidation by PGH synthase has been demonstrated in SHE cells (8). It is unknown to what extent estrogens are cooxidized *in vivo* in target tissues known to contain PGH synthase, e.g., uterus and breast. Taken together, our data show that DES and several DES analogues are metabolized and activated by PGH synthase, and a cor-

relation between *in vitro* conversion of the compounds and their transforming activity in SHE cells is observed.

PGH Synthase-Mediated Oxidation of Steroid Estrogens

Since several steroid estrogens and their catechol metabolites are known to transform cells in culture (3,27), their PGH synthase-catalyzed metabolism has been studied too. Estradiol (E₂), ethinyl-E₂, estrone (E₁), and estriol are metabolized by ARA-supplemented microsomal PGH synthase to a similar extent, and this is accompanied by the formation of protein-bound products; the catechols (e.g., 2-OH-E₂ and 2-OH-E₁) are oxidized more extensively than monophenolic estrogens (23). PGH synthase oxidizes by stepwise, one-electron abstraction: monophenolic estrogens are oxidized to phenoxy radicals, catechols to semiquinones and quinones; spectral detection of the (reactive) *ortho*-quinone intermediate is facilitated in incubations of 2-OH-E₂ with minute amounts of purified, highly active enzyme protein (28). Interestingly, PGH synthase also catalyzes the formation of catechols from steroid estrogens, as shown by tritium release from C-2 and C-4 (Table 1) and confirmed by product identification. The proposed mechanism is shown in Figure 3.

Table 1. PGH synthase-catalyzed regiospecific $^3\text{H}_2\text{O}$ release from estradiol and DES.

Structure and position of ^3H label	Radioactivity released as $^3\text{H}_2\text{O}$, % label, in	
	Complete incubations ^a	Controls ^b
E_2 	42.3 ± 2.3	2.7 ± 0.1
E_2 	29.0 ± 2.8	3.4 ± 0.1
DES 	6.5 ± 0.1	0.4 ± 0.0

^a Tritiated estrogens (10 and 50 μM) were incubated for 5 min at 37°C with microsomal PGH synthase (0.5 mg) and arachidonic acid (100 μM).

^b Incubations with heat inactivated protein (21,22).

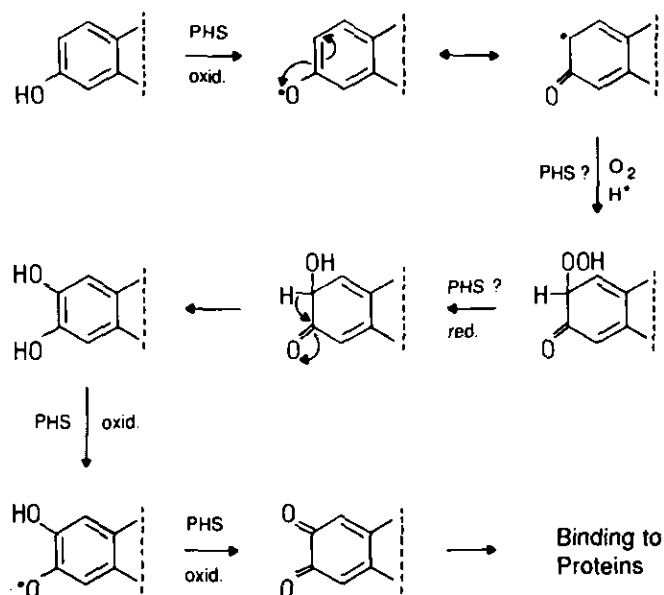


FIGURE 3. Mechanism of PGH synthase-catalyzed formation and oxidation of catechol estrogens. Initial oxidation of estradiol yields a phenoxyl radical (and its mesomeric keto form); this reacts with oxygen to give an *ortho*-hydroperoxide which is (non-)enzymatically reduced to the corresponding alcohol that tautomerizes to the catechol, which involves release of $^3\text{H}_2\text{O}$ from *ortho*-tritiated analogues. Subsequent catechol peroxidation proceeds via semi-quinone intermediates to *ortho*-quinones.

The formation of catechols from steroid estrogens is novel evidence for PGH synthase-mediated aryl hydroxylation: for suitable co-substrates such as E_2 it is a major reaction; for DES, which is oxidized mostly to Z,Z-DIES, it plays a minor role *in vitro* as has been studied using regiospecific tritium release from *ortho*-tritiated analogues (Table 1). Thus, PGH synthase can activate both stilbene and steroid estrogens via semi-quinone and quinone intermediates (*para*- for stilbenes, *ortho*- for steroids) (30).

Protein Binding of Reactive Estrogen Intermediates Generated by PGH Synthase Cooxidation

Using an *in vitro* system with purified PGH synthase and BSA as a competing nucleophile, we have recently shown that reactive intermediates of DES, HES, and steroid estrogens are released from the catalytic enzyme and bind to other protein(s) in addition to PGH synthase (21). Whether covalent modification of PGH synthase by estrogens in intact cells has biological implications is unknown at present. Proteins of the spindle apparatus have been suggested as critical cellular targets (26). When tubulin is included in incubations with PGH synthase, the reactive intermediates generated by

Table 2. PGH synthase cooxidation generates reactive intermediates of DES and estrone that bind preferentially to tubulin.

Incubation ^a	Radioactivity of estrogen bound to separated protein bands, dpm				
	A ^b	B ^c	PGH synthase	BSA	Tubulin
E ₁ , 5 μ M, - ARA PGH synthase-tubulin (1:1)	92 \pm 35	107 \pm 67	103 \pm 30	ND ^d	412 \pm 54
E ₁ , 5 μ M, + ARA PGH synthase-tubulin (1:1)	3808 \pm 186	733 \pm 130	1168 \pm 55	ND	7970 \pm 340
DES, 5 μ M, - ARA PGH synthase-tubulin (1:1)	81 \pm 41	44 \pm 21	182 \pm 47	ND	2158 \pm 293
DES, 5 μ M, + ARA PGH synthase-tubulin (1:1)	10120 \pm 2260	1531 \pm 204	2377 \pm 421	ND	6842 \pm 580
DES, 5 μ M, + ARA PGH synthase-tubulin (1:4)	15172 \pm 1335	2070 \pm 435	2069 \pm 108	ND	25925 \pm 2100
DES, 5 μ M, + ARA PGH synthase-BSA-tubulin (1:1:1)	10801 \pm 1876	1357 \pm 614	2294 \pm 335	1961 \pm 418	8649 \pm 186
DES, 5 μ M, + ARA PGH synthase-BSA-tubulin (1:4:1)	11198 \pm 1112	2058 \pm 1195	3573 \pm 1417	6886 \pm 1203	8779 \pm 1705

^aIncubations contained radiolabeled E₁ (121, 334 dpm/nmole) or DES (133,200 dpm/nmole) with or without arachidonic acid (ARA 50 μ M), PGH synthase (15 μ g/mL), and the other proteins in the ratios indicated.

^bA, sample load (crosslinked proteins).

^cB, region of microtubule-associated proteins.

^dND, not determined.

Table 3. Correlation between biological effects of several DES structural analogues and their PGH synthase-catalyzed metabolism *in vitro*.

Compound	Estrogenicity ^a	Transformation ^b	PGH synthase metabolism ^c
DES	High	+	+++
TF-DES	Medium	+	+++
DMS	Low	+ ^d	+++
HES	High	+ ^e	++
Z,Z-DIES	None	+	++
E,E-DIES	High	+ ^d	++
M-DES	Very low	+ ^f	+
DM-DES	Very low	- ^g	-
IA	Medium	?	+++
IB	Medium	?	Inhibits

^aPotency *in vivo* according to literature data (30,37,38).

^bSHE cell transformation data from McLachlan et al. (2) unless otherwise indicated.

^cData from Degen et al. (20,24). See text for further details.

^dResults not clearly positive when initially tested but clearly positive on reexamination (D. Schiffmann, personal communication).

^eResults not clearly positive when initially tested but clearly positive on reexamination (T. Tsutsui, personal communication).

^fResults not clearly positive when initially tested but clearly positive on reexamination (A. Wong, personal communication).

^gNot significantly different from untreated controls.

cooxidation of either radiolabeled DES or of estrone (E₁) bind preferentially to this protein, even in the presence of excess BSA (Table 2). The reactive intermediates also cause crosslinking of proteins and modify PGH synthase and microtubule-associated proteins covalently.

It has previously been shown that quinone intermediates of DES and 2-OH-E₂ (but not E₂) generated by oxidation with horseradish peroxidase (HRP) react preferentially with tubulin when proteins are added

after a few minutes of incubation time (29). The negative results with E₂ in the HRP system could be explained either by short-lived intermediates or by an inability of HRP to catalyze aryl hydroxylation to an extent similar to PGH synthase, which would yield less catechol precursor for subsequent activation.

Taken together, our data support the view that PGH synthase can provide (complete) metabolic activation of stilbene and steroid estrogens, even in the absence of monooxygenases that catalyze catechol formation. If tubulin is a critical target molecule (26,29) for reactive estrogen intermediates, these results support the contention that PGH synthase mediates the induction of aneuploidy by DES, DES-related compounds, and steroid estrogens, an effect that could be involved in neoplastic transformation.

Structure-Activity Relationships

At present, there is no conclusive evidence that PGH synthase-catalyzed metabolic activation is a decisive factor in the mechanism of action of carcinogenic estrogens. However, this proposal is supported by structure-activity relationships observed for a series of DES-related compounds. The structures, together with information on their metabolism by PGH synthase as well as transforming and estrogenic activity, are shown in Table 3. Apparently, there is a correlation between the ability to induce neoplastic transformation of SHE cells and *in vitro* PGH synthase-catalyzed metabolism: all derivatives found to transform SHE cells (irrespective of their estrogenicity) are also oxidized by PGH syn-

thase *in vitro* except for the dimethyl ether of DES (DM-DES), which is not metabolized.

Recently, closely related DES indanyl analogues have been found to differ in their interaction with PGH synthase: indenestrol A (IA) is cooxidized and activated like DES, whereas indenestrol B (IB) inhibits the enzyme (24). It is known that IA, an *in vivo* metabolite of DES and IB, its synthetic isomer, have very similar conformation and estrogenicity (30). IA is oxidized by PGH synthase (or HRP) to a *para*-quinone intermediate (20) that reacts with tubulin and inhibits its polymerization (26). In IB, one phenolic group is in the *meta*-position relative to the central double bond, precluding *para*-quinone formation. IB can be oxidized by peroxidases, most likely to a phenoxy radical, but it is not metabolized in a typical cooxidation reaction with arachidonic acid-supplemented PGH synthase because it inhibits the cyclooxygenase (24).

The different properties of these DES-indanyl analogues should facilitate future studies concerning the role of PGH synthase-mediated metabolism and of reactive intermediates in their genotoxic action. Thus, it is suggested that these compounds be included in studies assessing clastogenic and neoplastic potential.

Effects of Estrogenic Compounds on Prostaglandin Biosynthesis

The different interaction of IA and IB with PGH synthase, as indicated by concentration-dependent stimulation and inhibition of PGH synthase-cyclooxygenase activity, respectively (24), is also reflected in their effect on PGE₂ synthesis *in vitro*. IA (10 μ M) stimulates PGE₂ synthesis almost 3-fold above the control range (Fig. 2). The slight inhibitory effect seen at the highest concentration of IA is attributed to its antioxidant properties. This has also been observed with other compounds that are cooxidized; the compounds stimulate ARA conversion at low concentrations, but inhibit conversion at higher concentrations (20).

On the other hand, PGE₂ synthesis is inhibited by IB (Fig. 2). The IC₅₀ is not different when it is determined in the cyclooxygenase assay. IB inhibits with a similar IC₅₀, independent of the arachidonic acid concentration and apparently not by an antioxidant type but an indomethacinlike type of inhibition (24).

In this context, it is of interest that some antiestrogens, e.g., tamoxifen and related compounds, inhibit PGH synthase *in vitro* (31). Cooxidation of monophenolic or diphenolic estrogens, however, has been found to result in a stimulation of prostanoid synthesis *in vitro* (28). Their mode of interaction with PGH synthase and the ability of the enzyme to recognize apparently rather subtle structural differences of DES indanyl isomers is an interesting facet of this problem. It is hoped that X-ray structure analysis of the enzyme (32) will provide additional clues to the structural features involved. This applies also for other compounds that exhibit marked stereoselectivity as cyclooxygenase inhibitors but no

stereoselectivity as peroxidase-reducing substrates (33).

PGH synthase-catalyzed oxidation of estrogen co-substrates results in a nearly stoichiometric increase in prostanoid synthesis *in vitro* (28). Therefore, although estrogens can reach relatively high concentrations in target tissues, cooxidation seems unlikely to affect PG biosynthesis *in vivo* significantly unless estrogens induce arachidonic acid release and/or PGH synthase activity. Due to the complexity of the arachidonic acid cascade (11), we prefer to focus on effects of estrogens on PGH synthase itself that could result in a modulation of PG biosynthesis *in vivo*.

Future Perspectives

The production of PGs has been suggested as a prognostic marker for breast cancer patients (34) and is apparently related, not only to changes in fatty acid composition and availability, but also to increases in PGH synthase activity as determined in biopsy homogenates in the presence of nonlimiting amounts of substrate (35). Despite the well-known cyclic variations in PG biosynthesis in estrogen target tissues (17,18), very little is known as yet about the regulation and inducibility of PGH synthase by sex hormones. To develop this latter aspect further, polyclonal antibodies against highly purified ovine PGH synthase have been raised for immunoblot analysis. The method presently used detects 10 ng enzyme and has been applied successfully to quantitate differences in PGH synthase levels in human (HL-60) cells and in ram seminal vesicles of varying enzyme activity (36). Although further refinements are required before the hormonal regulation of PGH synthase in estrogen target tissues can be studied, the cross-reactivity of the PGH synthase antiserum with human PGH synthase (36) suggests that this approach could also be applied to the study of PGH synthase expression in human biopsy specimens.

Stimulating and fruitful discussions with Manfred Metzler (University of Kaiserslautern, FRG), John McLachlan and J. Carl Barrett (NIEHS, Research Triangle Park, NC) during past and recent collaborations are gratefully acknowledged. Bernd Epe kindly provided a sample of tubulin and critically read the manuscript. This work has been supported by the Deutsche Forschungsgemeinschaft by a grant to project B-3 of the Sonderforschungsbereich 172.

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